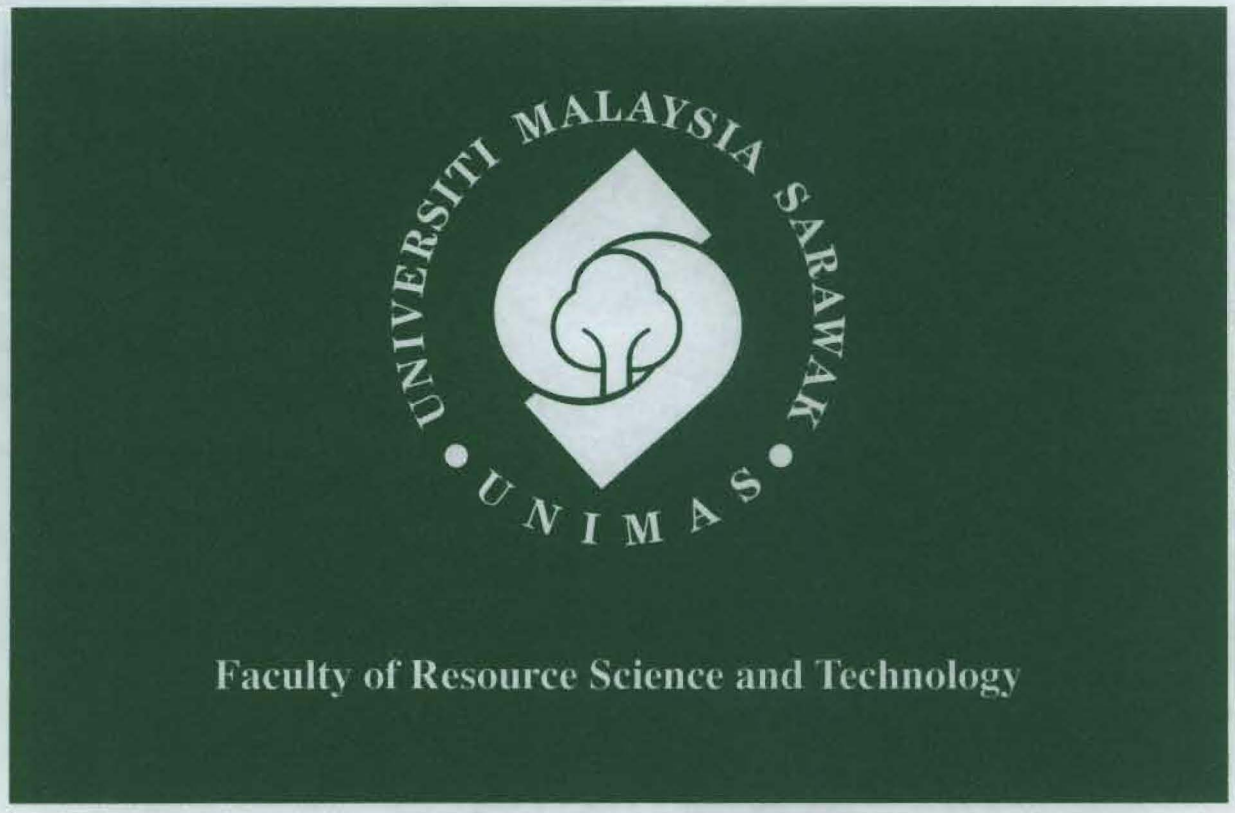

DETECTION AND ENUMERATION OF VIBRIO PARAHAEMOLYTICUS IN RAW SALAD VEGETABLES AT PRE-HARVEST LEVEL, RETAIL LEVEL AND DOMESTIC KITCHEN LEVEL

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Bachelor of Science with Honours
(Resource Biotechnology)
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Detection and Enumeration of *Vibrio parahaemolyticus* in Raw Salad Vegetables at Pre-harvest Level, Retail Level and Domestic Kitchen Level

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This project is submitted in partial fulfilment of the requirement for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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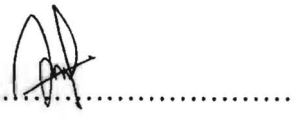
2012

Declaration

I hereby declare that this Final Year Project is based on my original work except for quotations and citations which have been properly acknowledged. I also declared that it has not been previously or concurrently submitted for any other degree in UNIMAS or other institutions.



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List of Abbreviations

V. parahaemolyticus	*Vibrio parahaemolyticus*
PCR	Polymerase Chain Reaction
MPN	Most Probable Number
spp	Species
V. cholera	*Vibrio cholera*
V. vulnificus	*Vibrio vulnificus*
TCBS	Thiosulfate Bile Salts Sucrose
APW	Alkaline Peptone Water
%	Percentage
°C	Degree Celsius
NaCl	Sodium chloride
mm	Millimeter
ml	Milliliter
g	Gram

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Detection and Enumeration of *Vibrio parahaemolyticus* in Raw Salad Vegetables at Pre-harvest Level, Retail Level and Domestic Kitchen Level

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ABSTRACT

The aim of this study was to detect and enumerate *Vibrio parahaemolyticus*, the leading cause of gastroenteritis illness in human, from raw salad vegetables at pre-harvest level, retail level and domestic kitchen level in Kuching, Sarawak. In this study, a combination of Most Probable Number supplemented with Polymerase Chain Reaction method were applied to detect the presence of *Vibrio parahaemolyticus*, harboring the *tox*R gene and to enumerate their density in the samples. Based on the real event in the domestic kitchen, stimulation was designed and conducted to result a useful data and information from the consumption of raw salad vegetables. With the MPN supplemented with PCR technique, out of 360 samples, none of them were detected to carry the *tox*R gene. However, the result highlighted that the consumption of raw salad vegetables can be a transmission route for *V. parahaemolyticus;* this can possess risks to the consumers.

Keywords: *Vibrio parahaemolyticus*, MPN, PCR, *tox*R gene, vegetables

ABSTRAK

Kajian ini bertujuan untuk mengesan dan mengira kehadiran *Vibrio parahaemolyticus* yang dikatakan menjadi punca penyakit gastroenteritis kepada manusia akibat pemakanan sayur-sayuran mentah pada peringkat sebelum penuaian, pemasaran dan dapur domestik di Kuching, Sarawak. Dalam kajian ini, kombinasi teknik Most Probable Number dan Polymerase Chain Reaction digunakan untuk mengesan kehadiran *V. parahaemolyticus* yang mempunyai gen *tox*R serta mengira densiti dalam sampel. Berdasarkan situasi sebenar di dapur, satu simulasi dihasilkan dan dijalankan bagi menghasilkan data serta maklumat yang berguna untuk mengenalpasti risiko daripada pemakanan sayur-sayuran mentah. Dengan penggunaan kombinasi teknik MPN dan PCR, daripada 360 sampel yang telah diuji, tiada satu sampel yang menunjukkan mereka mempunyai gen *tox*R. Walau bagaimanapun, keputusan kajian mendapati pemakanan sayur-sayuran secara mentah mampu menjadi punca kepada penyebaran *V. parahaemolyticus* di mana ia mampu memberikan risiko yang berbahaya.

Kata kunci: *Vibrio parahaemolyticus*, MPN, PCR, gen *tox*R, sayur-sayuran

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Meldrum *et al.* (2009) reported that the two recent large outbreaks in the United Kingdom demonstrated the significant health problems that could arise from consumption of contaminated salads. Another study of the prevalence of pathogens in vegetables was reported by Little *et al.* (2007), in which they found *Listeria monocytogenes* in ready-to-eat mixed salads in the UK. Local studies of other microorganisms found in vegetables were also reported recently (Chai *et al.*, 2008; Learn-Han *et al.*, 2009; Ponniah *et al.*, 2009). However, the foodborne outbreaks of *Vibrio parahaemolyticus* gastroenteritis did not happen frequently in the USA and Europe but very common in Japan (Kenneth and Amy, 2001). In recent year, *Vibriosis* had emerged to be an important bacterial disease that causes human intestinal and extra-intestinal infections all over the world (Garcia *et al.*, 2009 and Harth *et al.*, 2009).

Vibrio parahaemolyticus, the leading cause of seafood-associated gastroenteritis in the United States (Joseph *et al.*, 2005) is known as a common foodborne pathogen in Asia (Lee *et al.*, 2008), and has been reported to be the cause for 20-30% food poisoning cases in Japan (Gopal *et al.*, 2005). Recent foodborne outbreaks throughout the world have been intensively linked to consumption of fresh fruit, vegetables and unpasteurized juices (Gorny, 2006). In Malaysia, an outbreak in Kedah in 2003 was reported to be caused by *V. parahaemolyticus* linked to 'kerabu tauge', a local dish mixed with vegetables (Mohamad *et al.*, 2006). Tunung

et al. (2010) reported that the vegetable samples from the supermarkets and wet markets in Selangor, Malaysia were positive for the pathogenic species of *V. parahaemolyticus*. The *V. parahaemolyticus* cases had been reported frequently been attributed to the consumption of raw or uncooked seafood, or the ingestion of contaminated water, but to the best of our knowledge there have been limited reported study on *V. parahaemolyticus* in raw vegetables (Tunung *et al.*, 2010).

There are three levels have been studied in this research; pre-harvest level, retail level and domestic kitchen level. The vegetables samples for the pre-harvest level were collected from the farms at Kota Samarahan and Kuching, Sarawak. Meanwhile for the retail level, the samples were purchased from Everise Supermarket, Unaco Supermarket, Stutong wet market and Kota Samarahan wet market. For the domestic kitchen level, the simulation was carried out in the Microbiology Laboratory, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak.

Nowadays, Polymerase Chain Reaction (PCR) has been proven to be useful in detecting pathogens in food samples rapidly and accurately. *V. parahaemolyticus* possesses a regulatory gene, *tox*R which is present in all strains and PCR based on *tox*R was reported to be specific for *V. parahaemolyticus* which is useful for confirmation of this species (Kim *et al.*, 1999; Gopal *et al.*, 2005). PCR methods have been developed for specific detection of *V. parahaemolyticus*, however they are limited to qualitative determination of the organism unless they are used in conjunction with Most Probable Number (MPN) procedure (Su and Liu, 2007). The MPN method is commonly used to measure the concentration of the target microbe in samples (Kaysner and DePaola, 2004). However, apart from labor-intensive and

time-consuming, a major disadvantage of MPN is that the thiosulphate-citrate-bile salts-sucrose agar (TCBS) used in the method cannot differentiate *V. parahaemolyticus* from some strains of *V. vulnificus* or *V. mimicus* (Su and Liu, 2007). There are many presence epidemiology reports and qualitative studies of prevalence related to food poisoning caused by *V. parahaemolyticus* in seafood but there is a lack of quantitative or enumeration studies on it that have been published (Lee *et al.*, 2008) in local settings, especially on *V. parahaemolyticus* in raw vegetables.

Therefore, the aims of this study were to enumerate and detect the numbers of *V. parahaemolyticus* in vegetables that are usually eaten raw in Malaysia. Besides, it also to stimulate the common procedures practiced in the handling of vegetables in domestic kitchens. Different washing procedures were applied to determine effectiveness of the decontamination steps.

1.2 Objectives

Therefore, the objectives of this research are as the following:

1. To enumerate the number of *V. parahaemolyticus* in raw salad vegetables in Kuching, Sarawak at pre-harvest level, retail level and domestic kitchen level by using MPN method.
2. To detect the presence of *V. parahaemolyticus* in raw salad vegetables in Kuching, Sarawak at pre-harvest level, retail level and domestic kitchen level by using PCR method.

CHAPTER 2

LITERATURE REVIEW

2.1 The genus *Vibrio*

Vibrio spp. is Gram-negative, facultatively anaerobic, motile, curved rod with a single polar flagellum (FEHD, 2005) and its genus belongs to the family *Vibrionaceae* which consists of at least 34 recognized species (HPA, 2007a). There are three types of *Vibrio* species which commonly cause foodborne outbreak around the world; *V. parahaemolyticus, V. cholerae and V. vulnificus.* They account for the significant proportion of human infections as most of these vibrios secrete enterotoxins in food, water or in gastrointestinal tract (Nishibuchi and DePaola, 2005). The vibrio can grow at a pH range from 5.6 to 9.6 but grows best at pH 7.6 to 8.6 (R. Sakazaki, 2003).

2.2 *V. parahaemolyticus*

V. parahaemolyticus, a Gram-negative, halophilic bacterium that inhabits warm estuarine waters is the common cause of seafood-associated bacterial gastroenteritis in the worldwide (Bilung *et al.*, 2005). *V. parahaemolyticus* is serotyped by its lipopolysaccharide (LPS), somatic O and capsular polysaccharide K antigens (Kenneth and Amy, 2001). The optimum temperature for the growth of *V. parahaemolyticus* is 37°C and at this optimum temperature, the bacterium has generation times of 8 to 9 minutes in water and 12 to 18 minutes in seafood

(Montville and Matthews, 2008). Unlike *V. cholerea,* optimum growth occurs in media containing 0.5mol/L sodium chlorides and it produces catalase and cytochrome oxidase (Oliver and Kaper, 1997). The incubation period of *V. parahaemolyticus* infection is nearly 24 hours followed by explosive, watery diarrhea accompanied by nausea, vomiting, abdominal cramps and sometimes fever (Talaro, 2009). Even though the gastroenteritis caused by *V. parahaemolyticus* infection is often self-limited, it may cause septicaemia which is life-threatening to immunocompromised individuals (Su and Liu, 2007). As non-pathogenic vibrios can be present in food and environmental samples, total *Vibrio* counts are not indicative enough for the presence of pathogenic vibrios (Tunung *et al.,* 2010). Hence, the presence of virulence genes are always considered as current markers of pathogenicity in *V. parahaemolyticus* (Robert-Pillot *et al.*, 2004).

2.3 Raw salads vegetables

According to a study published in American Scientist magazine November/December issue (Western Farm Press, 2007), the increase in outbreak of food poisoning in mainly due to the people eating more fresh fruit, vegetables, salad and more meals outside of home. Trends in many countries towards eating more raw fresh or lightly cooked vegetables to preserve taste and heat labile nutrients may also increase the likelihood of foodborne infections (Erdogrul and Sener, 2005). Okafo *et al.* (2003) reported the presence of *Escherichia coli, Vibrio* spp. and *Salmonella* spp. in raw vegetables harvested from soils irrigated with contaminated steams in Nigeria. Fresh fruit and vegetables eaters are at risk because the produce may be grown on the contaminated soil. They are also subjected to the contamination during the pre-harvest processing. After harvesting, contamination may include bacteria transmitted by

people whose hands or tools are dirty with human or animal feces or their own feces (Western Farm Press, 2007).

The occurrence of *V. parahaemolyticus* in vegetables among Malaysian citizen is high since the vegetables are often consumed raw as 'ulam' and as accompaniment to popular dishes such as 'nasi lemak' (Ponniah *et al.*, 2009). The raw salad vegetables usually purchased from the supermarkets or wet markets which both setting provide a large variety of food types for sale, from poultry, meat, seafood, fresh vegetables and fruits. The differences of both settings are based on the hygiene condition and the turnover of the food products, in which supermarkets displayed a more hygienic conditions, slower turnover, with foods mostly packaged before sold, meanwhile wet markets displayed poor hygienic condition, food non-packaged and freshly sold but with fast turnover of products (Chai *et al.*, 2007; Ponniah *et al.*, 2009).

Vegetables and fruits particularly those eaten raw and without peeling, have been demonstrated to be vehicles for transmission of a range of microorganism (Rodina, 2007). Raw vegetables are normally exposed to microorganisms and condition which enhance microbial multiplication during growth, transportation and preparation before consumption. The risk of human infections can be reduced by preventing contamination, controlling growth and removing or killing pathogens by washing or treating raw fruits and vegetables with sanitizers (Fraatz *et al.*, 2010).

2.4 Detection and Quantification of *Vibrio parahaemolyticus*

Polymerase Chain Reaction (PCR) is used to detect the specific genes in the organism meanwhile the MPN is a serial dilution tests that measured the concentration of target microbe in a sample with an estimate which particularly useful for low concentrations of organism (<100 cell/g), especially in milk and water and for those foods whose particular matter may interfere with accurate colony count. However, the major disadvantage of MPN is that the thiosulphate-citrare-bile salts-sucrose agar (TBCS) used in the method cannot differentiate *V. parahaemolyticus* from some strains of *V. vulnificus* or *V. mimicus* (Su and Liu, 2007). This is where the PCR will complement the MPN procedure, through accurate confirmation of the presence of *V. parahaemolyticus* in the sample tested (Tunung *et al.*, 2010). The primer sequences used in this study is shown in Table 2.1.

Primer	Primer sequence	Length	Amplicon size	Reference
*tox*R4 (forward)	5'-GTCTTCTGACGCAATCGT-3'	20bp	369bp	Kim *et al.*, 1999
*tox*R7 (reverse)	5'-ATACGAGTGGTTGCTGTCATG-3'	21bp		

Table 2.1: Primer sequence

CHAPTER 3

MATERIALS AND METHODS

3.1. Pre-harvest level

3.1.1 Sample collection

The collections of the samples were carried out at two vegetables farm chosen randomly in Kota Samarahan, Kuching, Sarawak. Fresh vegetables were obtained and placed in sterile plastic bags. All samples were transported to the laboratory immediately and being analyzed within 24 hours of sample collection. The samples collected were tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*).

3.1.2 Sample preparation

The sampling method performed in this study was based on the Bacteriological Analytical Manual standard method (Kaysner and DePaola, 2004) with modification according to the procedure by Chai *et al.* (2007). Ten gram portion of each vegetable sample was placed in a stomacher bag with added 90 ml of APW with 3% NaCl followed by incubation at 37°C for 18-24 hour.

3.3.3 Culturing methods

A loopful of culture from each MPN tube was streaked onto CROMagar™ Vibrio. Then, the plates were incubated at 37°C for 18-24 hour. The *V. parahaemolyticus* isolates were confirmed by using specific PCR targeting *tox*R gene of *V. parahaemolyticus.*

3.1.4 Detection and Quantification of *Vibrio Parahaemolyticus*

For three-tube Most Probable Number (MPN) analysis, a 100 fold and a 1000 fold dilutions of the stomacher fluid were prepared. Portions of each dilution were transferred into three tubes, with each tube containing 1ml, and then the tubes were incubated at 37°C for 18-24 hours. After incubation, a loopful of culture from each tube was streaked onto CROMagar™ Vibrio, which is a new chromogenic agar for isolation of *V. parahaemolyticus* containing substrates for beta-galactoside (Hara-Kudo et al., 2001). Prior to PCR, the MPN tubes were first preceded to DNA extraction. The primer sequences use as shown in Table 2.1.

3.1.5 DNA extraction

The MPN tubes were subjected to DNA extraction. The procedures were performed using the boil cell method (Su and Liu, 2007) with slightly modification. A 1 ml portion of each MPN broth were subjected to centrifugation at 13,400 x g for 1 min and the pellet were resuspended in 500 µl of sterile distilled water. The mixture were boiled for 10 min and then

immediately cooled at -20°C for 10 min before recentrifuged it again at 13,400 x g for 3 min. The supernatant then were used in PCR for detection of *tox*R gene which is specific for *V. parahaemolyticus* (Tunung *et al.*, 2010).

3.1.6 PCR assay

The method was based on Zulkifli *et al.* (2009) with some modification on the amount of the PCR reagents for optimization. Sterile distilled water was used as negative control and the positive control contains the *tox*R gene. Table 3.1 and Table 3.2 shows amount of PCR reagents and the PCR parameter that has been used respectively.

Table 3.1: PCR reagent and amount

Reagents	Amounts
Distilled water	12 µl
PCR buffer	4 µl
dNTP	1 µl
$MgCl_2$	3 µl
***tox*R Foward**	1 µl
***tox*R Reverse**	1 µl
DNA template	2 µl
Total	25 µl

Table 3.2: PCR parameter

	Temperature (°C)	Time (min)
Predenaturation	96 °C	5 min
Denaturation	94 °C	1 min
Annealing	63 °C	1.5 min
Extension	72 °C	1.5 min
Final extension	72 °C	7 min

3.1.7 Gel electrophoresis

The PCR products were run on 1.2% agarose gel in 1x Tris Borate EDTA (TBE). The ladder that has been used was 100 bp. 5 µl of PCR products were mixed with 1 µl of loading dye then were loaded into the sample wells. The gel was run at 90 volt for 1 hour. Then the gel was stained in the ethidium bromide for 15 minutes. Then it was visualized and photographed under UV transilluminator.

3.2 Retail level

3.2.1 Sample collection

Everise Supermarket, Unaco Supermarket, Stutong wet market and Kota Samarahan wet market were chosen for this study. The differences between supermarkets and wet markets are based on their holding time and the way food items are processed prior to sale. The hygiene level of the vegetables samples source were also observed. During collection, all the samples were transferred to sterile plastic bags for transportation and being analyzed immediately on arrival to the laboratory. The temperature of the surrounding during sampling was recorded. The samples to be collected are tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*).

3.2.2 Sample preparation

The sample preparation was performed as described in section 3.1.2.

3.2.3 Culturing Methods

The culturing procedure was performed as described in section 3.1.3.

3.2.4 Detection and Quantification of *Vibrio parahaemolyticus*

The procedure was performed as described in section 3.1.4.

3.2.5 DNA Extraction

The procedure was performed as described in section 3.1.5.

3.2.6 PCR assay

The procedure was performed as described in section 3.1.6

3.2.7 Gel electrophoresis

The procedure was performed as described in section 3.1.7